Polymerase Chain Reaction Protocol

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Author

- Erica Suchman

Information

History

The polymerase chain reaction (PCR) was developed in 1983 by Dr. Kary Mullis while working for Cetus Corporation. In 1993, he received the Nobel Prize in Chemistry for this important contribution that revolutionized molecular biology (3, 4, 7, 8). The technique can be used to amplify DNA sequences from any type of organism. It has been adapted over the years to allow amplification of RNA samples, as well as quantification of the amount of DNA or RNA in a sample. The isolation of a thermal stable DNA polymerase (*Taq*) from an archaebacteria isolated from a geothermal vent in Yellowstone National Park allowed the reaction to be carried out in a single closed tube driven by varying temperatures.

Purpose

The PCR is an extremely useful technique for specific in vitro amplification of nucleic acids. It has a large number of applications. The utility of PCR comes from the very small amount of starting material required. Manipulation of the specificity can be achieved by simply varying length and nucleotide sequence of primers and annealing temperature. This can be of particular importance in medical diagnosis when an infectious agent is present in low numbers. The PCR is also an important diagnostic tool for many genetic diseases and chimerism testing for bone marrow transplants. Furthermore, it has played a pivotal role in the analysis of microbial species, such as amplifying and sequencing 16S rRNA in order to understand the phylogenetic relationships among different bacterial species.

Theory

For purified DNA in an appropriate reagent mixture, the procedure for PCR is as follows (Fig. 1). The temperature is raised to 92 to 98^{o}C, causing the DNA strands to separate or denature. This step often lasts 1 minute. The temperature is then lowered and the two primers, of approximately 20 nucleotides each (conventionally one is called the forward primer and the other is called the reverse primer), are annealed to opposite strands of the DNA. (RNA requires an initial reverse transcription step to create a double-stranded cDNA template.) This step often lasts 1 minute. The temperature is raised to the optimum for a polymerase from a thermophilic bacterium, the bacterium is usually *Thermus aquaticus (Taq)* at a temperature of 72^{o}C, and

replication starts from the 3' OH of the primers producing copies of the DNA.

The *Taq* polymerase has no proofreading function (3'-5' exonuclease activity), therefore is prone to generate errors during DNA synthesis. (Other thermostable archeal DNA polymerases such as *Pfu* which has 3'-5' proofreading function can be used for certain PCR applications.) The size of the target nucleic acids to be amplified determines the duration of this step. In general, 1 Kb of DNA takes 1 minute to amplify. The temperature is again raised to 92 to 98^{o}C, causing the DNA strands to separate, then lowered to allow new primers to attach to each of the 4 strands created in the last reaction, and raised to 72ºC for the primer extension. As this three-step cycle repeats, target nucleic acids are amplified. The temperature used during the annealing of primers must be optimized for each individual primer set (3, 4, 7, 8). A rough estimate of the expected optimal temperature can be determined by analyzing the G and C content of the primers. However, using a gradient thermal cycler, one can experimentally determine the best annealing temperature. A gradient thermal cycler allows a slightly different temperature to be achieved in each sample, allowing one to try many different annealing temperatures during a PCR experiment. If a gradient thermal cycler is not available, one can use the following equation to determine the melting point (T_m) of the primer sets. This T_m approximation can be used as the annealing temperature for the first attempts and adjusted if necessary.

C x (number of A's and T's in the primer) (1, 2, 6; http://www.promega.com/biomath/calc11.htm#disc)

The *Taq* polymerase is stable during the DNA separation and denaturation step and is therefore not denatured and able to begin a new cycle of synthesis. The process is repeated for 20 to 30 cycles so that additional copies arise exponentially, i.e., in a chain reaction. In addition, 40 to 50 cycles can be run in many applications, where additional*Taq* polymerase can be added after 20 to 25 cycles. After amplification, the PCR product, sometimes called an amplicon, is analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain and compared to known-sized molecular markers for production of bands of the correct size.

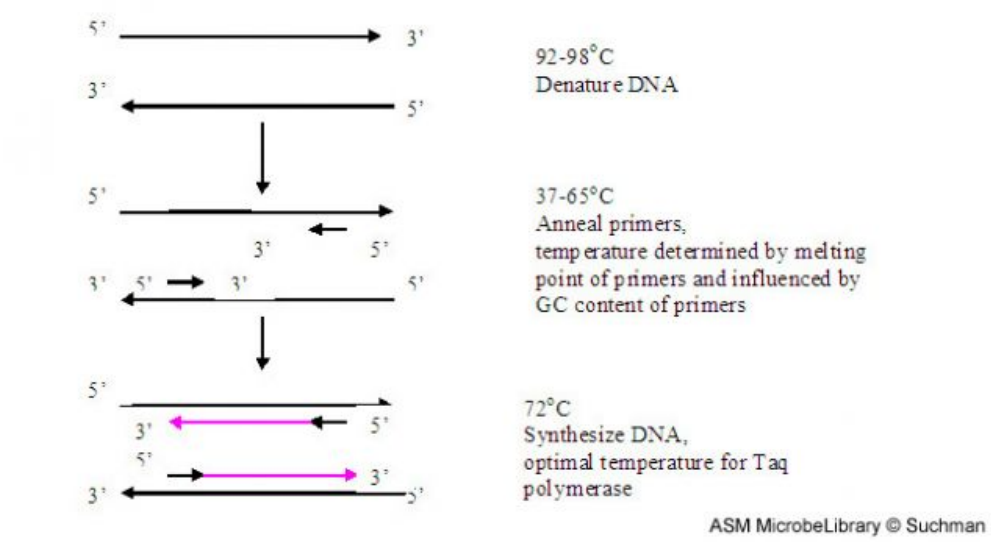

FIG. 1. Simplified illustration of PCR amplification.

Although PCR uses a DNA polymerase to amplify DNA of interest, RNA of interest can be detected by inserting a pre-PCR step that creates a complementary DNA (cDNA) using the retroviral enzyme reverse transcriptase (RT). Primers complementary to either the specific RNA sequence or the poly(A) tail can be used to begin production of the cDNA.

It is an interesting historical note that when Dr. Mullis developed the PCR procedure, the thermal-stable DNA polymerase *Taq* had not yet been isolated. Therefore, after each denaturation step, DNA polymerase had to be added to each tube, necessitating opening each tube and making crossover contamination a serious issue. The isolation of *Taq* polymerase allowed the entire reaction to occur in a closed tube.

Standard PCR allows one to determine if target nucleic acids are present but is not very useful for quantifying samples. If quantification is desired, one usually performs real time quantitative PCR (developed in the early 2000s) which requires the addition of an internal fluorescently-labeled probe that hybridizes between the two primers (Taqman RT PCR) (Fig. 2) or the use of double-stranded DNA-binding fluorescent dyes such as SYBR green (9). The amount of PCR product is usually quantified using a fluorescence detector, and the number of cycles of amplification required to cross a threshold fluorescence value (cycle threshold or CT) is determined by the computer and manipulated by the user. The fewer the number of cycles required to cross the threshold, the more target nucleic acids are present in the sample. The CT values of unknown samples can be compared to CT values of known concentration standards to quantify the amount of target nucleic acids in the samples. However, again if one wants to quantify RNA, the RNA must first be reverse transcribed to cDNA and which is then used to perform real time PCR.

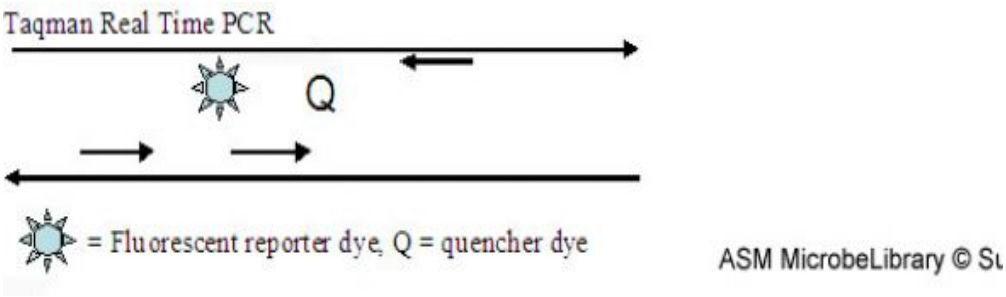

FIG. 2. Real time PCR.

The Taqman probe is complementary to sequences between the two primers used to amplify the DNA. This internal Taqman probe contains a 5' fluorescent reporter dye and a 3' quencher dye that disrupts (or quenches) the detectable signal from the 5' fluorescent reporter dye when it is in close proximity via fluorescence resonance energy transfer (FRET). As *Taq* polymerase polymerizes the DNA, its 5' exonuclease activity will cleave the 5' fluorescent reporter dye from the Taqman probe liberating it. As it floats away from the 3' quencher dye, its fluorescence will be detected by the detector.

SYBR green or related double-stranded DNA dyes work by binding to double-stranded DNA as it is amplified. Although this method is cheaper and easier than Taqman PCR, these dyes have no specificity for correctly amplified product and will bind to misprimed PCR products and can give artificially high readings.

PROTOCOL

Polymerase chain reaction

Usually 20 to 50 µl total in volume and will include the following:
X µl, 0.1 to 1 µg of genomic DNA or cDNA, ~0.1µg should be sufficient for plasmid DNA (5)
10X PCR buffer to give a final concentration of 1X
4 mM dNTP mix (dCTP, dATP, dGTP, dTTP) to give a final concentration of 0.2 mM
Both the forward and reverse primer added at a final concentration of 0.1 µM to 1 µM of each primer
1 unit/µl *Taq* polymerase
H_2O (DNA and DNase free) to bring volume to 20 µl to 50 µl
An example 20 µl reaction
1 µl of dsDNA template (~0.1 µg)
2 µl of 10X buffer
1 µl of 4 mM dNTP mix
1 µl of 10 µM forward primer to a final concentration of 0.5 µM
1 µl of 10 µM reverse primer to a final concentration of 0.5 µM
1 µl of 1 unit/µl *Taq* polymerase
13 µl of water
Combine the reagents in the 0.5-ml tube or the 0.2-ml PCR tube. Be sure to keep the reagents on ice. Tap tube gently to mix and spin briefly in microcentrifuge to get all contents to bottom, then place on ice until ready to load in thermocycler. If thermocycler does not have a heated lid, layer thin film of mineral oil over mixture to prevent evaporation

during cycling.

Upon completion of PCR, hold samples at 4°C. Prepare the DNA for loading by addition of 1/10 volume stop-loading buffer (contains EDTA, glycerol, and bromphenol blue). Analyze by gel electrophoresis and be sure to include size markers in at least one well on the same gel.

Example results

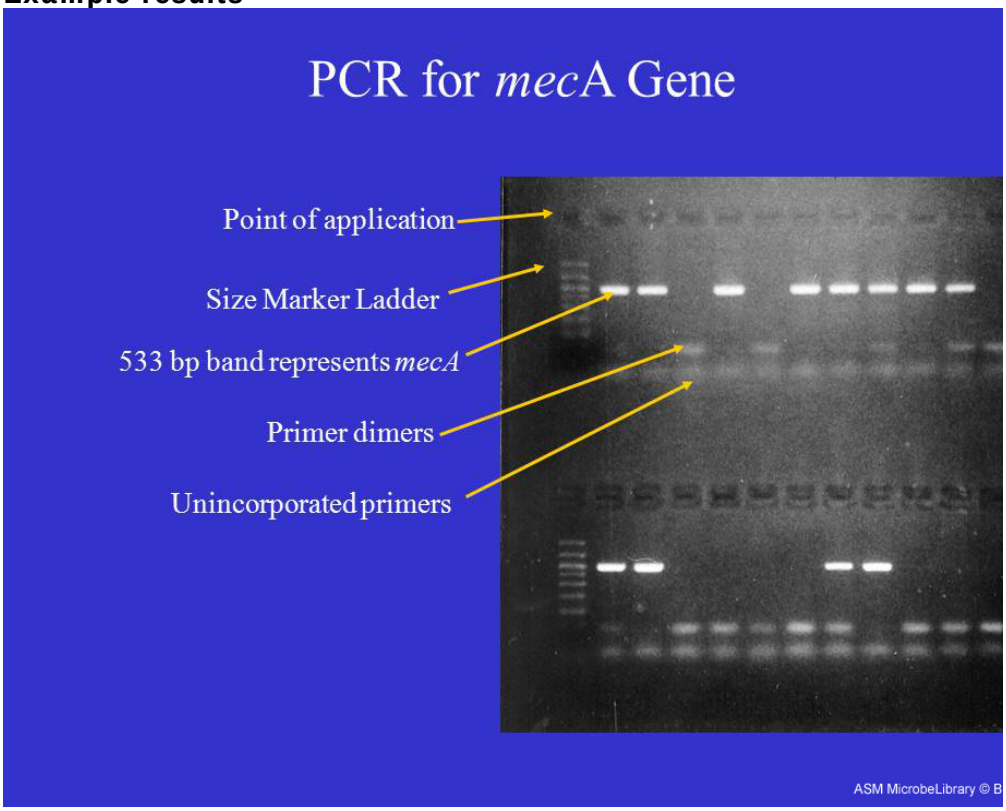

FIG. 3. Example PCR gel electrophoresis agarose gel demonstrating a 533 bp amplicon as well as primer dimers and unincorporated primers. (Rebecca Buxton, University of Utah)

Example typical thermal cycler program

Step 1: 92 to 98°C, 30 seconds to 1 minute
Step 2: optimal annealing temperature of primers, 37 to 65°C, 30 seconds to 1 minute
Step 3: 72°C, 30 seconds to 1 minute
Repeat steps 1 to 3 for 20 to 30 times to accumulate enough amplified target DNA to be visualized on a gel.
Step 4: 4°C holding of sample until analysis by gel electrophoresis

This graph shows a typical temperature program for a PCR reaction describing what is happening at each step:

A. Denaturation (strands separate)

B. Annealing (primers anneal to complementary sequences of the template DNA)

C. Elongation (Facilitated by the polymerase, complementary dNTP's synthesize a new DNA strand.)

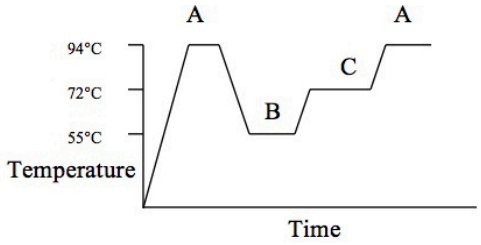

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FIG. 4. Typical temperature program for a PCR reaction. (Rebecca Buxton, University of Utah)

Potential problems (5; http://www.highveld.com/pages/pcr-troubleshooting.html)

1. Positive and negative controls must be used and run every time.
2. Too little primer will result in inadequate amplification. Too much primer will increase the probability that primer dimers (self-binding of primer to primer rather than primer to template) will form.
3. Self-complementary sequences in the primers allow primer dimer formation.
4. Too little or too much *Taq* polymerase will result in no PCR product or excess nonspecific products. Use the amount of *Taq* recommended by the vendor.
5. Inadequate or old dNTPs will result in no PCR product.
6. Inadequate or old *Taq* polymerase will result in no PCR product.
7. Too much or too little target DNA will result in no PCR product or excess nonspecific products.
8. Poor primer design will result in no PCR product or excess nonspecific products. When developing primers for your PCR reaction, consider the following variables: primer length (17 to 22 nt), T_m (45 to 65°C), and product length. Avoid primers that self-anneal. The frodo.wi.mit.edu/primer3 website can be used to help design primers.
9. Incorrect annealing temperature will result in no PCR product or excess nonspecific products.
10. Impurities such as phenol or too much salt will result in no PCR product or excess nonspecific products.
11. False positives due to contamination, often from DNA-contaminated water or other reagents.
12. Due to the ability to amplify very low amounts of target template, carry-over contamination of PCR product is a substantial issue. Strict aseptic technique is essential.
13. The use of dUTP as a substitute for dTTP can prevent carry-over contamination from previous amplifications. PCR amplification using dUTP will generate uracil-containing PCR products that are suitable for most standard applications. To prevent these amplified products from contaminating other PCR amplifications performed afterward in the same laboratory, before a PCR amplification one can treat the PCR premix with the enzyme uracil-N-glycosylase, UNG (also referred to as UDG) to excise uracil from any uracil-containing PCR products from previousamplifications so they will not be amplified in the current

reaction, thereby preventing false positives (9). Using dUTP for PCR and pretreating PCR with UDG have become standard practice in many clinical diagnostics labs.

14. Further precautionary measures to avoid carry-over contamination and false positives may include the use of positive displacement pipettes, cotton plugged tips, master mixes, and UV treatment of samples before the addition of*Taq* polymerase and DNA to nick any contaminating DNA. It is also important to have designated areas of the lab where PCR reactions are set up, preferentially separated in space from the areas where PCR reactions are analyzed by gel electrophoresis. Many of these are standard practices in clinical and research laboratories.

15. Mispriming of primers leading to bands of unexpected sizes. This can be reduced by searching data bases with potential primers to be sure they do not have homology to any known genes.

16. Unoptimized Mg^{+2} concentration will result in no PCR product or excess nonspecific products. The *Taq* enzyme manufacturers usually include buffers of varying Mg^{+2} concentrations for scientists who wish to perform optimization experiments, but classroom instructors will probably want to use established, preoptimized procedures.

17. Unoptimized annealing temperature will result in no PCR product or excess nonspecific products. If no bands are observed the following are the most likely causes: missing PCR component, no DNA, too little DNA, wrong annealing temperature, inadequate number of cycles, *Taq* polymerase not working, old NTPs, wrong primer set or concentration, impurities in DNA sample.

18. If multiple bands are seen, likely causes include: low annealing temperature, too high Mg^{2+} concentration, contaminations, primer dimer formation, too much primer.

19. If smears are seen, likely causes include: DNA degradation, no primers, or missing components.

20. If PCR products of the wrong size are seen, likely causes include: incorrect primer design, template mutations, contamination, mispriming of primer, incorrect annealing temperature.

21. Primer design for real time PCR differs depending on whether Syber green or Taqman are used, but in all cases will differ from standard PCR primer design.

22. Commercial kits are available from many sources and can be used to reduce variability in reagent quality.

23. Standard *Taq* polymerase has its limitations. For error-free products modified *Taq* polymerases with proofreading ability should be employed. For products greater than 5 kb and less than 40 kb, long extender *Taq*polymerases are available commercially.

24. For some applications, especially using tested and robust primers, a boiled DNA prep using colonies picked with a sterile toothpick without isolation of DNA also works and offers a quick and cheap way to do PCR in teaching laboratories. This can save time and effort for PCR reactions that are known to work well, but should be tested ahead of time to be sure it will work.

25. Protocols that do not use harsh and dangerous chemicals, e.g., phenol, are available online. They produce sufficient quality to enable most routine PCR applications.

26. For isolation of DNA from gram-positive organisms, adding a lysozyme step is often helpful.

27. Many commercial providers offer premade kits to isolate genomic and/or plasmid DNA. The cost is higher than doing your own protocol but offers a higher consistency and purity of DNA product.
28. PCR can be used for many applications such as comparison of eukaryotic and prokaryotic organisms by 16S and 18S rDNA, idenfication of allelic variation, identification of aerobic versus anaerobic bacteria in water, and production of large quantities of product for cloning or sequencing.
29. Spectrophotometer readings at 260/280 of primers can be used to determine the DNA concentration; take the absorbance and multiply it by the dilution and 50 µg/µl to determine the total µg/µl.

RECIPE

Materials for PCR

DNA or cDNA sample
0.50-ml microcentrifuge tube
P20 micropipeter (pipetman)[a]
Box of micropipeter tips (yellow)[a]
Beaker of ice
Reverse primer and forward primer (Note: these can be in many different concentrations) 0.1 µM to 1 µM
Mixture of 4 mM each of 4 deoxynucleoside triphosphates
10X PCR buffer (1X = 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM TrisHCl, pH 9.0, 1% Triton X-100)
1 unit/µl *Taq* DNA polymerase
Mineral oil (if thermocycler does not have hot lid to reduce condensation)
Thermocycler

[a]Note: in a research or diagnostic lab, the use of positive displacement pipettes and cotton stuffed tips is recommended to prevent crossover contamination. In a teaching lab these may be prohibitively expensive, and standard pipetmen and tips can be used. Students should be informed that in situations where avoidance of crossover contamination is critical, the use of standard pipetmen and yellow tips is not recommended.

Materials for reverse transcription

RNA extracted by the guanidinium isothiocyanate-phenol-chloroform method
MMLV reverse transcriptase (RNase H)
Reverse transcriptase buffer containing 10 mM DTT, 10 mM dNTPs, 40 unit RNasin in 20 µl
Reverse primer (complement of nucleotides in the sample to be amplified)

Procedure for preparing RNA for reverse transcriptase PCR

1. RNA extraction. To sample to be analyzed (tissue or cells can be used) add 2.0 ml of guanidine thiocyanate solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7, 0.5% Sarkosyl, 100 mM 2-mercaptoethanol). Add the following sequentially, with mixing after each addition: 200 µl 2M sodium acetate, 2.0 ml H_2O-saturated phenol, and 400 µl $CHCl_3$:isoamyl alcohol (49:1). Incubate samples on ice for 15

minutes, then centrifuge at 10,000 rpm for 20 minutes at 4°C. Precipitate RNA from the aqueous phase by addition of an equal volume of isopropyl alcohol, wash with 2.4 ml of 3 M sodium acetate, wash with 70% ethanol, dry, and dissolve in 10 µl of DEPC-treated H_2O.

2. Reverse transcription. Usually a small volume of total RNA (1.0 µl) is incubated at 70°C for 10 minutes with 15 pmol of the reverse transcription primer in a total volume of 10 µl. The mixture is cooled to room temperature, and 67 units of Superscript RT (BRL, Gaithersburg, MD) in 10 µl 2X RT buffer are added (final concentration of buffer = 1X, final volume = 20 µl). Reverse transcription was carried out at 37°C for 1 hour.

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

COMMENTS AND TIPS

Using plasmids as the DNA template almost always ensures you will have successful results as using a pure template works very efficiently. Using good quality DNA preparations and well-designed primers, amplification of high copy templates usually works quite reliably as well.

It is easy to do "mock" PCR reactions. In my virology course, students are given tubes labeled as if they contain PCR reagents when in fact all tubes contain water. They set up the reactions, run them in the PCR machine, and some of their samples are spiked with PCR products from my research laboratory. They then run their samples on a gel during the next lab period. Some of the samples are positive, some are negative, and some contain fragments of incorrect size. Students then have to analyze samples from everyone in the class and interpret all data, including what could be the cause of fragments of unexpected size. Fragments of incorrect size are included to allow students to explore the reasons why mispriming of primers might occur, such as poor design, too low an annealing temperature, polymorphisms, etc.

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REVIEWERS

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Rebecca Buxton
University of Utah, Salt Lake City, UT

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Jackie Reynolds
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Amy Siegesmund
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CONTRIBUTERS

The following contributed to the Comments and Tips section at the ASM Conference for Undergraduate Educators 2011.

Participating contributors:

Afsane Arvand
Mount St. Mary's College, Los Angeles, CA

Tatiana Ascencio
Universidad Dr. José Matías Delgado, San Salvador, El Salvador

Warner Bair
Lone Star College—CyFair, Cypress, TX

Christine Bezotte
Elmira College, Elmira, NY

Michael Chase
Montgomery College, Rockville, MD

Madhusudan Choudhary
Sam Houston State University, Huntsville, Texas

Brooks Crozier
Roanoke College, Salem, VA

Doreen Cunningham
Saint Augustine's College, Raleigh, NC

Amy Warenda Czura
Suffolk County Community College, Riverhead, NY

Christine De Stefano
Community College of Baltimore County, Baltimore, MD

Deven Dilinger
Community College of Baltimore County, Baltimore, MD

Uchechi Ekwenye
Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State, Nigeria

Sara Fruehling
St. Olaf College, Northfield, MN

Joel Funk
John Brown University, Siloam Springs, AR

Diane Gibson
Hazard Kentucky Community Technical College, Hazard, KY

Woodnard Givens
Northern Virginia Community College, Sterling, VA

Brinda Govindan
San Francisco State University, San Francisco, CA

Deborah Harbour
College of Southern Nevada, Las Vegas, NV

Kai (Billy) Hung
Eastern Illinois University, Charleston, IL

Zeynep Ceren Karahan
Ankara University School of Medicine, Ankara, Turkey

Dave Kushner
Dickinson College, Carlisle, PA

Garry Marley
Oklahoma State University, Stillwater, OK

Betsy Martinez-Vaz
Hamline University, Saint Paul, MN

Katherine Mixter Mayne
William Woods University, Fulton, MO

Gordana Mircevska
Institute of Microbiology and Parasitology, University Saints Cyril and Methodius, Skopje, Macedonia

Amy Miller
University of Cincinnati, Raymond Walters College, Cincinnati, OH

Heather Miller
North Carolina State University, Raleigh, NC

Hilda Nyati
National University of Science and Technology, Bulawayo, Zimbabwe

Jennifer O'Connor
Rose-Hulman Institute of Technology, Terre Haute, IN

Paul O'Leary
San Juan College, Farmington, NM

Juana Ortellado-Canese
Universidad Nacional de Asunción, Asuncion, Paraguay

Rafael Ovalle
Brooklyn College, Brooklyn, NY

Samantha Parks
Georgia Institute of Technology, Atlanta, GA

Joanne Rampersad
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Helen Schneider
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David Singleton
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David Straus
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Sardha Suriyapperuma
Connecticut College, New London, CT

Amy White
Virginia Western Community College, Roanoke, VA